PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/74

A1

(11) International Publication Number: WO 91/09131

(43) International Publication Date: 27 June 1991 (27.06.91)

(21) International Application Number: PCT/DK89/00298

(22) International Filing Date: 20 December 1989 (20.12.89)

(71) Applicant *(for all designated States except US)*: VALIO FINNISH COOPERATIVE DAIRIES ASSOCIATION [FI/FI]; Kalevankatu 56, SF-00181 Helsinki (FI).

(71)(72) Applicants and Inventors: WESSELS, Stephen [DK/DK]; Gammel Strandvej 422C, DK-3060 Espergærde (DK). JOSEPHSEN, Jytte [DK/DK]; Magnoliavej 34, DK-2000 Frederiksberg (DK). VOGENSEN, Finn [DK/DK]; Kirsebærbakken 5, DK-3400 Hillerød (DK). NIELSEN, Egil, Waagner [DK/DK]; Smedegade 12, DK-3500 Kirke Værløse (DK).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)* : VON WRIGHT, Atte [FI/FI]; Lohkaretie 13, As 10, SF-70700 Kuopio (FI). TYNKKYNEN, Soile [FI/FI]; Tiistiläntie 6 D 33, SF-02230 Espoo (FI).

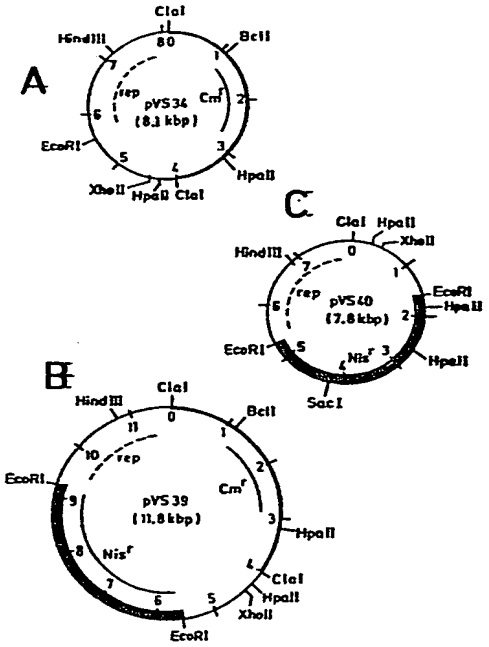
(74) Agent: PLOUGMANN & VINGTOFT; Sankt Annæ Plads 11, DK-1250 Copenhagen K (DK).

(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE*, DE (European patent)*, DK, ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US.

Published

With international search report.

(54) Title: CLONING VECTOR FOR USE IN LACTIC ACID BACTERIA



(57) Abstract

A food-grade innocuous cloning vector for use in lactic acid bacteria comprises a replication region with a narrow host range, preferably the replication region of a 28 kbp plasmid from *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* SSD207, and a bacteriocin resistance determinant, preferably the nisin resistance determinant from plasmid pSW211 obtained from *Lactococcus lactis* subsp. *lactis* 10.084, as a selectable marker. With this vector it is possible to construct genetically engineered products (i.e. organisms or chemical products) which are safe enough to be a part of human food. Neither those bacteria in which the vector can replicate, nor the DNA which makes up the vector offer any hazard to human health.

* See back of page

SDOCID: <WO 9109131A1>

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FI**	Finland	**ML**	Mali
AU	Australia	**FR**	France	**MN**	Mongolia
BB	Barbados	**GA**	Gabon	**MR**	Mauritania
BE	Belgium	**GB**	United Kingdom	**MW**	Malawi
BF	Burkina Faso	**GN**	Guinea	**NL**	Netherlands
BG	Bulgaria	**GR**	Greece	**NO**	Norway
BJ	Benin	**HU**	Hungary	**PL**	Poland
BR	Brazil	**IT**	Italy	**RO**	Romania
CA	Canada	**JP**	Japan	**SD**	Sudan
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SE**	Sweden
CG	Congo			**SN**	Senegal
CH	Switzerland	**KR**	Republic of Korea	**SU**	Soviet Union
CI	Côte d'Ivoire	**LI**	Liechtenstein	**TD**	Chad
CM	Cameroon	**LK**	Sri Lanka	**TG**	Togo
DE	Germany	**LU**	Luxembourg	**US**	United States of America
DK	Denmark	**MC**	Monaco		
ES	Spain	**MG**	Madagascar		

:DOCID: <WO 9109131A1>

Cloning vector for use in lactic acid bacteria

Introduction

The present invention relates to a food-grade inocuous cloning vector for use in dairy lactic acid bacteria.

More specifically, the invention concerns the man-made combination of two pieces of DNA (i.e., carried out by genetic manipulation) which are derived exclusively from two different Lactococcus strains (formally termed Streptococcus), both of which can be used in starter cultures for dairy products. These bacteria are completely inocuous and are eaten in dairy products, which is why the invention (DNA) also is considered to be completely inocuous when it exists in the bacteria of starter cultures.

The result of the combination of the two different pieces of DNA has the following implications:

(a) The invention, i.e. the cloning vector, carries a marker for resistance to nisin which is genetically selectable in the lactococci. Nisin is a bacteriocin which is produced by several strains of Lactococcus. The most fundamental function of the marker gene of any cloning vector is to reveal the presence of the vector in cells transformed with it. The marker gene serves this function in particular in the decisive step of all genetic manipulation in which the mixture of the vector DNA and the new gene in question, hopefully cloned onto the vector, is transformed into the new host cells. At this step it is absolutely crucial to be able to actually select for the presence of the vector in the relatively few cells which have received it among the hundreds of millions of cells involved in the experiment.

(b) The cloning vector has a replication gene with a very narrow host-range, or spectrum of function. A replication gene makes it possible for a segment of DNA (e.g., a chromosome or a plasmid) to be duplicated at each cell division. When a replication gene is said to have a narrow host-range, it can only be expressed ("understood") in closely related species of bacteria. In the case of the present invention, the replication gene is expressed only in Lactococcus, Lactobacillus and Streptococcus salivarius subsp. thermophilus, but not in Bacillus, or E. coli. Thus, the vector can be expected to remain in the bacterial species into which the scientist in a given research-and-development laboratory originally placed it.

Background of the invention

The lactic acid bacteria have been a safe part of the human diet for thousands of years and can therefore be considered as food-grade bacteria. These bacteria include the groups Lactococcus, Pediococcus, Lactobacillus, Leuconostoc and Bifidobacterium.

The dairy industry, as well as other biotechnological industries, are at present showing an increasing interest in obtaining the ability to manipulate these bacteria genetically. This would allow these industries to optimize the utility of the bacteria in the food products in which these organisms traditionally have been utilized, as well as allow adapting them to novel biotechnological processes.

In order to manipulate the lactic acid bacteria genetically and still preserve their food-grade quality, it is necessary to construct cloning vectors which themselves are of food-grade quality. For this reason all the functions making up such vectors, e.g. marker and replication

genes, should also derive from the lactic acid bacteria.

(a) The replication function

The invention is directed to the construction of a vector, which contains only DNA of lactococcal origin. The replication function of the vector has been taken from a large lactococcal plasmid. The present vector is able to replicate in lactococci, in some lactobacilli and at a very low frequency in Staphylococcus aureus, but not in Escherichia coli or Bacillus subtilis. This narrow spectrum of function restricts the spreading of genes which have been cloned into the vector, into other bacterial species.

Lactococcal dairy starter strains contain a wide variety of plasmids with sizes ranging from about 2 Kbp (Kilobase pairs) to more than 80 Kbp. Large plasmids are often associated with such phenotypes as lactose fermentation, proteolysis, conjugation, phage resistance, etc. (for a review, see McKay, L.L., Antonie van Leeuwenhoek J. Microbiol. 49, 259-274 (1983)). The majority of the lactococcal plasmids, however, are cryptic. Small cryptic plasmids, such as pWV01 and pSH71, have been used by several research groups for vector construction by marking the plasmids with antibiotic resistance genes derived from plasmids of other gram positive bacteria (DeVos, W.M., Neth. Milk Dairy J. 40, 141-154 (1986), DeVos, W.M., FEMS Microbiol. Rev. 46, 281-295 (1987), Gasson, M.J. and Anderson, P.H., FEMS Microbiol. Lett. 30, 193-196 (1985), Kok, J., van der Vossen, J.M. and Venema, G., Appl. Environ. Microbiol. 48, 726-731 (1984) and von Wright, A., Tynkkynen, S. and Suominen, M., Appl. Environ. Microbiol. 53, 1584-1588 (1987)).

The lactococcal cloning vectors constructed so far share an ability to replicate in a wide range of bacterial

hosts, such as Escherichia coli, Bacillus subtilis, Staphylococcus aureus, and some of the lactobacilli. Although this property is very useful for research purposes, it will be unacceptable for food-grade vectors aimed at genetic manipulation of actual dairy starter strains. It would be undesirable if recombinant plasmids were to spread into other genera of bacteria in the dairy or farm environments or in the digestive tract of humans or domestic animals.

(b) The selectable marker

As a selectable marker on the invention is employed the lactococcal gene for resistance to the bacteriocin nisin.

Individual strains of the lactic acid bacteria produce bacteriocins and are, thus, inherently resistant to these compounds. The genes coding for these resistances could be used as the selectable markers on food-grade cloning vectors.

The best studied bacteriocin so far is nisin, which is produced by various strains of Lactococcus lactis subsp. lactis (Hurst, A., Adv. Appl. Microb. 27, 85-123 (1981) and Kaletta, C. and Entian, K.-D., J. Bacteriol. 171, 1597-1601 (1989)). Nisin is a peptide of 34 amino acids which probably owes its bacteriocidal effect to interference with the function of the cytoplasmic membrane of Gram positive bacteria by acting on the phospholipid components of the membrane (Henning, S., Metz, R. and Hammes, W.P., Int. J. Food Microbiol. 3, 121-134 (1986) and Sahl, H.-G., Kordel, M. and Benz, R., Arch. Microbiol. 149, 120-124 (1987)).

A number of research groups have attempted to adapt the lactococcal gene for resistance to nisin (nis^r) to use as

a food-grade selectable marker. Using the strain ATCC 11454, both Gonsalez and Kunka, Appl. and Environmental Microbiology 49, 627-633 (1985) and Steele and McKay, Appl. Environ. Microbiol. 51, 57-64 (1986) obtained evidence from conjugation experiments and from plasmid curing that this strain carries a plasmid encoding sucrose fermentation (Suc^+), nisin production (Nip^+) and nisin resistance (Nis^r). However, efforts to isolate the DNA involved in these phenotypes did not meet with success.

For each of 8 different strains of L. lactis subsp. lactis, Gasson, FEMS Microbiol. Letters 21, 7-10 (1984), was also able to provide evidence by conjugation for the coexistence on a single plasmid of genes for Suc^+, Nip^+ and Nis^r. But here, too, for all strains the plasmid DNA responsible for these traits proved to be elusive.

In L. lactis subsp. lactis ME2, Klaenhammer and Sanozky, J. Gen. Microbiol. 131, 1531-1541 (1985) found a genetic linkage between bacteriophage resistance, lactose fermentation (Lac^+) and Nis^r. Although a spontaneous curing, as well as a conjugation, localized these genes to a 64 kbp (kilopase pair) plasmid, it was not possible to isolate the DNA of the plasmid in question. Neither was it possible to select directly for Nis^r transconjugants receiving the plasmid due to a high frequency of spontaneous resistant mutants.

The linkage on a single plasmid of nisin resistance and resistance to bacteriophage attack was also observed by McKay and Baldwin, Appl. Environ. Microbiol. 47, 68-74 (1984), in L. lactis subsp. lactis biovar. diacetylactis DRC3. They, too, were able to show by conjugation and spontaneous curing that these genes were encoded by a 64 kbp plasmid, pNP40. In 1988 Froseth et al., Appl. Environ. Microbiol. 54, 2136-2139 (1988), fortuitously discovered

the close, physical linkage on pNP40 between the *nis*^{r} gene and a replication function. This DNA fragment was isolated and successfully propagated in lactococcal cells. Unfortunately, as with the gene from the strain ME2, this nisin resistance determinant was not suitable for direct genetic selection of the trait.

It has now surprisingly been found possible to isolate and clone a nisin resistance determinant which is well suited for use as a primary selectable marker in vectors.

Summary of the invention

The present invention thus relates to novel cloning vectors whose DNA is exclusively of lactococcal origin and containing (a) a region of replication with a very narrow spectrum of function and (b) a nisin resistant determinant as a genetically selectable marker. The invention further comprises methods for construction of such vectors, intermediate vector plasmids for use in the construction of said vectors, recombinant plasmids between such vectors and DNA fragments cloned using them, and lactic acid bacteria transformed with these vectors.

The novel vectors were developed using Lactococcus lactis subsp. lactis MG1614, into which - in the first stage - the replication region of a cryptic plasmid from Lactococcus lactis subsp. lactis biovar. diacetyllactis SSD207 is cloned using the chloramphenicol resistance gene from a streptococcal plasmid as the selection marker. The replication gene has a very narrow host-range, and will thus be expressed only in closely related species of bacteria. Into this plasmid, an EcoRI fragment from plasmid pSW211, coding for nisin resistance, and originally cloned from the lactococcal plasmid pNis, is cloned in order to obtain a nisin-chloramphenicol doubly resistant plasmid. From

this plasmid the streptococcal chloramphenicol resistance region is subsequently eliminated. The resulting plasmid contains, as stated above, only lactococcal DNA, and it has a wide range of application.

The vector can be used for instance for food-grade gene manipulations, e.g. for genetic engineering of lactococci for use in foods and pharmaceuticals, for research on and production of nisin, for stabilization of starter cultures etc. In addition, the proposed inocuousness of the vector can be useful in biotechnology industries to ensure safety in the workplace environment.

Brief description of the drawings:

Figure 1 demonstrates the derivation of the constructions employing the cloned replication function: pVS34, pVS39 and pVS40.

Figure 2 shows the restriction maps of the originally cloned nisin resistance determinant and its EcoRI sub-clone. A. pSW211. B. pSW221.

The restriction sites indicated on the DNA derived from pNis (thick lines) were mapped using the particular plasmid on which they are positioned. The location of the nisin resistance determinant on pSW221 (cross-hatched area) is based on the observation that deletion of the smaller EcoRI - SacI fragment results in inactivation of the resistance.

Figure 3 demonstrates the derivation of cloned nisin resistance determinant by its Southern hybridization to plasmids used in this invention.

A. Agarose gel (0.8%). B. Hybridized membrane. Probe consisted of the EcoRI Nis^r fragment of pSW221.

Electrophoresed plasmid DNA:

lane 1, total plasmid DNA of strain 10.084, not restricted;

lane 2, total plasmid DNA of strain 10.084, restricted with HindIII (partially digested);

lane 3, total plasmid DNA of strain 10.084, restricted with EcoRI (partially digested);

lane 4, pNis, not restricted;

lane 5, pNis restricted with HindIII;

lane 6, pVS2, restricted with HindIII;

lane 7, pSW211, not restricted;

lane 8, pSW211, restricted with HindIII;

lane 9, pSW211, restricted with EcoRI;

lane 10, pSW211, not restricted;

lane 11, pSW221, restricted with EcoRI (partially digested);

lane 12, pVS34, restricted with EcoRI (partially digested);

lane 13, pVS39, restricted with EcoRI;

lane 14, lambda DNA, restricted with HindIII [molecular weight standard in base pairs: 23,130; 9416; 6557; 4361; 2322; 2027; 565 (barely visible in agarose gel)]

Arrows indicate I: HindIII Nis^r fragment; II: EcoRI Nis^r fragment.

Figure 4 shows the effect of nisin on exponentially growing cells with and without nisin resistance determinant.

Cultures of LMO230(pVS2) (O) and LMO230(pSW211) (□) were diluted into GM17 broth to give A_{600} 0.05. At about A_{600} 0.2 they were challenged with 10 µg pr. ml nisin, which was repeated 1 ½ h later.

The construction of the vectors is described in further details in the following examples, which are merely illustrative and not intended to limit the scope of the invention in any way.

Thus it should be understood that the invention is not limited to specific details of these examples. Various modifications and variations are possible within the scope of the invention. For instance, further screening of lactococcal plasmids will reveal replication regions that have different effects on plasmid copy number, host range, incompatibility and mobilization. By subsequently joining such fragments to the nisin resistance marker (or some other food-grade acceptable selectable gene), a series of cloning vectors useful for the food industry and related industries can be generated.

In the examples, it is shown how the replication region of a 28 kpb cryptic plasmid from Lactococcus lactis subsp. lactis biovar. diacetylactis SSD207 was cloned into L.

lactis subsp. lactis MG1614 using the chloramphenicol resistance gene from the streptococcal plasmid pGB301 as the selection marker. The resulting 8.1 kbp plasmid was named pVS34. The 4.1 kbp ClaI fragment representing lactococcal DNA in pVS34 contains the following unique restriction sites: HindIII, EcoRI, XhoII, and HpaII, of which the last three can be used for molecular cloning. A region necessary for replication is located within 2.5 kbp fragment flanked by the EcoRI and ClaI restriction sites.

Likewise, in the examples it is shown how the genetic determinant for resistance to nisin encoded on the plasmid pNis, originating from Lactococcus lactis subsp. lactis 10.084, was cloned into L. lactis subsp. lactis MG1614 following restriction and ligation of the pNis DNA to the plasmid pVS2 and transformation selection for nisin resistance. The resulting plasmid, termed pSW211, is 12.7 kbp in size.

A 3.7 kbp EcoRI fragment from plasmid pSW211, coding for nisin resistance, was cloned into the EcoRI site of pVS34 to obtain a nisin-chloramphenicol double resistance plasmid pVS39. From this plasmid the streptococcal chloramphenicol resistance region was subsequently eliminated. The resulting plasmid, pVS40, contains only lactococcal DNA.

Materials and Methods

Bacterial strains, plasmids, media and culture conditions

Lactococus lactis subsp. lactis biovar. diacetylactis SSD207 is isolated from a dairy starter culture. In gel electrophoresis it shows at least 12 plasmid bands ranging in size from 2 to more than 30 kbp, von Wright et al., Lett. Appl. Microbiol. 2, 73-76 (1986). L. Lactis subsp. lactis 10.084 was obtained from the strain collection

maintained at the Danish Government Research Institute for Dairy Industry. This strain carries 5 or 6 plasmids, as illustrated in Figure 3A, lane 1. Recipient strains and different plasmids used in the transformation and cloning experiments are detailed in table I. M17 medium (Terzaghi, B.E. and Sandine, W.E., Appl. Microbiol. 29, 807-813 (1975); Difco, East Molesey, UK), supplemented with either 0.5% lactose (for SSD207, 10.084 and strains carrying the plasmid pNis) or glucose (for the rest of the strains) was used throughout this work with L. lactis subsp. lactis and Staphylococcus aureus. Escherichia coli and Bacillus subtilis were grown in L-broth or -agar, as defined in Maniatis et al, Molecular Cloning: A laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1982) MRS-medium (DeMan, J.C. et al., J. Appl. Bact. 23, 130-135 (1960) was used with Lactobacillus plantarum. The incubation temperature was 32°C for the lactococci, 30°C for L. plantarum and 37°C for the others.

The strain MG1614 was obtained from M. Gasson, a derivative of L. lactis subsp. lactis NCDO 712, which had been cured of its plasmids and prophage following several rounds of protoplast curing. The strain LMO230, received from L.L. McKay, is described as a derivative of L. lactis subsp. lactis C2, which has been cured of its plasmids and prophage as a result of treatment with nitrosoguanidine and ultraviolet radiation. The mixed strain starter cultures BOL11 and D1 are both produced by Chr. Hansen's Laboratory Ltd., Hørsholm, Denmark, and both are used routinely for production at the Danish Government Research Institute for Dairy. BOL11, widely employed in the production of cheese, is a Direct Vat Set (freeze-dried) starter reported to contain approximately 1 - 5% L. lactis subsp. lactis, 70 - 75% L. lactis subsp, cremoris, and 2 - 5% Leuconostoc cremoris (Chr. Hansens's Laboratorium Ltd., Declaration of mesophilic lactic ferment cultures).

Media and culture conditions

For the detection of the lactose- or sucrose-fermentation phenotype, the bromcresol purple indicator of McKay et al., Appl. Microbiol. 23, 1090-1096 (1972), was supplemented with 1% lactose or sucrose, respectively. The proteinase phenotype was tested by inoculating one colony into 10 ml autoclaved reconstituted skim milk supplemented with 0.5% glucose and 0.1% litmus. After incubation for 24 h at 30°C, a clone causing decoloration of the litmus and coagulation of the milk was judged to be proteinase-positive. To determine whether strains were nisin producers, the agar flip-over method of Scherwitz et al., Appl. Environ. Microbiol. 45, 1506-1512 (1983), was employed with LMO230 as the indicator organism. For routine detection and selection of the nisin resistance determinant, nisin was added at 100 µg pr. ml to solid media. The antibiotics chloramphenicol (Cm) and erythromycin (Em) were added to final concentrations of 5 and 2.5 µg pr. ml, respectively.

For measurement of growth in broth, A_{600} was monitored on a Spectronic 501 spectrophotometer (Milton Roy Corp.), and viable cells were counted [colony forming units (cfu) pr. ml] by plating appropriate dilutions on GM17 agar.

To determine single-cell resistance to nisin, approximately 10^5 cfu of an exponentially growing culture in GM17 broth were spread onto freshly made GM17 plates and GM17 plates containing concentrations of nisin ranging from 10 to 3000 µg per ml. The plates were incubated for 24 h, after which the approximate number of colonies on each plate was judged relative to the plates without nisin.

Table I

Strain	Relevant phenotype[a]	Description and reference
Lactococcus lactis subsp. lactis biovar. diacetylactis SSD207	Lac^+ Prt^-	Lett. Appl. Microbiol. 2, 73-76 (1986)
Lactococcus lactis subsp. lactis 10.084	Nip^+ Nis^r Lac^+ Suc^+ Prt^+	The strain collection of the Danish Government Research Institute for Dairy Industry
MG1614	Nis^s Rif^r Str^r	Plasmid-free derivative of L. lactis subsp. lactis NCDO 712 ; J. Bacteriol. 154, 1-9 (1983)
LMO230	Nis^s	Plasmid free derivative of L. lactis subsp. lactis C2; Appl. Environ. Microbiol. 32, 45-52 (1976)
MG1614(pNis)	Nip^+ Nis^r Lac^+ Suc^- Prt^+ Rif^r Str^r	This invention (second-round transformant by plasmids of strain 10.084)
MG1614(pSW211)	Nis^r Cm^r Em^r Rif^r Str^r	
MG1614(pSW221)	Nis^r Em^r Rif^r Str^r	
MLO230(pSW211)	Nis^r Cm^r Em^r	
MLO230(pSW221)	Nis^r Em^r	
E. coli AB259	thi	Mol. Gen. Genet. 127, 47-55 (1973)
B. subtilis 3G18	Ade, Met, Trp	Gerard Venema, the University of Groningen, the Netherlands
S. aureus RN451		Richard Novick, the Public Health Research Institute of the City of New York, Inc., New York
Lb. plantarum 755	Contains a cryptic plasmid of about 37 kbp	The strain collection of the Research and Development Centre of Valio Finnish Cooperative Dairies

Table I (Cont'd)

Plasmid	Phenotype	Size (kbp)	Description and Reference
pBR322	Amp^r, Tet^r	4.3	Gene 2, 95-113 (1977)
pVC5	Amp^r, Cm^r, Tet^r	8.4	The 4.1 kbp ClaI fragment containing the chloramphenicol resistance gene of pGB301, Mol. Gen. Genet. 184, 115-120 (1981), cloned to the single ClaI site of pBR322 (S. Tynkkynen, M.Sc. Thesis, 1985, University of Helsinki, Helsinki, Finland
pVS1	Cm^r	4.5	The 2.8 kbp ClaI-HpaII fragment of pVC5 cloned on the 1.7 ClaI fragment of pSH71; J. Bacteriol. 154, 1-9 (1983) (S. Tynkkynen, M.Sc. thesis)
pNis	Nip^+ Nis^r Lac^+ Suc^- Prt^+	46	This invention
pVS2	Cm^r Em^r	4.9	Appl. Environ. Microbiol. 53, 1584-1588 (1987)
pSW211	Cm^r Em^r Nis^r	12.8	This invention (pVS2::Nis^r/HindIII)
pGKV10	Em^r	4.6	Appl. Environ. Microbiol. 50, 540-542 (1985)
pSW221	Em^r Nis^r	8.4	This invention (pGKV10::Nis^r/EcoRI)
pVS34	Cm^r	8.1	This invention
pVS39	Cm^r, Nis^r	11.8	This invention
pVS40	Nis^r	7.8	This invention

[a] Abbreviation; Nip^+, Nisin-production positive; Nis^r, nisin resistant; Lac^+, lactose fermenting; Suc^+, sucrose fermenting; Prt^+, proteinase-production positive; Rif, rifampin; Str, streptomycin; Cm, chloramphenicol; Em, erythromycin; Tet, tetracycline; s, sensitive; r, resistant Ade, Met, Trp, adenine methionine tryptophan auxotroph.

Nisin solutions

Nisin was contained in the product Nisaplin, which is manufactured by Aplin and Barrett Ltd., Trowbridge, Wilts., England. According to the manufacturer, the major components of Nisaplin are milk proteins (17.12%), carbohydrate (5.9%) and NaCl (74.7%), plus nisin at 1026 units pr. mg (Aplin and Barrett, pamphlet accompanying product). In the present patent application references to concentrations of nisin are synonymous with Nisaplin concentrations. Stock solutions of nisin were prepared by dissolving Nisaplin at 10 mg pr. ml in 0.02 N HCl and were in no further way sterilized.

Frequency of spontaneous nisin resistant mutants

The frequency of spontaneous nisin resistance mutants was determined for the plasmid-free lactococcal strains MG1614 and LM0230. Cells of each strain grown GM17 broth were harvested by centrifugation, resuspended in one-tenth the original volume sterile distilled water and plated in the appropriate dilutions on GM17 agar containing 0, 100 and 1000 µg nisin pr. ml. Colonies were counted after incubation for 24 h and 48 h.

Frequency of occurence of nisin resistant organisms in dairy starter cultures

In order to mimic dairy practice with respect to reactivation of each freeze-dried starter product, 0.158 g and 1.01 g of BOL11 and D1, respectively, were asceptically weighed, inoculated into 1 L of freshly autoclaved reconstituted skim milk and incubated at 30°C for 3 h. Then dilutions were plated on GM17 plates either without nisin or containing 100 and 1000 µg pr. ml nisin. The plates were incubated for 24 h before the number of colonies was

accessed.

Plasmid DNA isolation and molecular cloning

For both small-scale and preparative isolation of plasmid DNA, the method of Anderson and McKay, Appl. Environ. Microbiol. 46, 549-552 (1983), was used. For preparative purposes the lysate was further purified by cesium chloride-ethidium bromide density gradient ultracentrifugation. All restriction mapping was conducted using gradient-purified DNA. Concentrations of DNA were evaluated according to the agarose plate method suggested by Maniatis et al., Supra.

All cloning was done employing DNA isolated either from strain MG1614 or LM0230, and the ligation mixtures in each case were transformed into MG1614. Then small-scale preparations of transformant DNA were retransformed into either MG1614 or LM0230. Restriction endonucleases, calf intestine phosphatase, and T4-DNA ligase (all from Boehringer Mannheim GmbH) and Gene Clean (Bio 101, Inc.) were employed according to the instructions of the manufacturer.

Transformation

In the construction of pVS34, pVS39 and pVS40, L. lactis subsp. lactis MG1614 protoplasts were transformed as described in Appl. Environ. Microbiol. 50, 1100-1102 (1985). The method of Mandel and Higa (J. Mol. Biol. 53, 159-162 (1970)) was used to transform E. coli AB259. Chloramphenicol (5 µg/ml) was used as the selection agent with MG1614, and, either alone or together with ampicillin (at concentrations of 5 and 50 µg/ml, respectively), with E. coli. The nisin-resistance phenotype of chloramphenicol-resistant MG1614 transformants was checked on

agar containing 500 µg/ml of a nisin-preparation.

For the isolation of the nisin resistance determinant protoplast transformation was essentially conducted according to von Wright et al., Appl. Environ. Microbiol. 50, 1100-1102, with the following modifications: Strains to be transformed were diluted 20 times from a fresh overnight culture into GM17 broth and incubated for 3 h at 30°C. This culture was then diluted 1000 times into ice cold GM17 broth, kept on ice overnight, and then grown at 30°C for about 5 h before harvesting at A_{600} 0.4. As the osmotic stabilizer 0.5 M sucrose [household granulated (pearl) sugar, The Danish Sugar Corp., Copenhagen, Denmark] was employed, and all water was freshly glass distilled. Protoplasting proceeded in GM17S-SAM (M17 broth and 0.5 M sucrose, autoclaved; 0.5% glucose, 1 mM Mg acetate, 4 mM NH_4 acetate, pH 7.0, filter sterilized). A 10 ml culture volume of the strain MG1614 was treated with 4 mg pr. ml lysozyme (Sigma, St. Louis, MO, grade I) at 37°C for 15 min, while 10 ml of LM0230 was protoplasted in 1 mg pr. ml lysozyme at 37°C for 15 min. Hereafter all manipulations were carried out at ambient temperature. Protoplasts were washed once in the transformation buffer SMMC (0.5 M sucrose, 20 mM Na_2 maleate, 20 mM $MgCl_2$, 50 mM $CaCl_2$, pH 6.5 filter sterilized), and finally resuspended in 500µl SMMC.

Each transformation sample contained DNA made up to 10 µl in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA), 10 µl 2 x SMMC, 50 µl protoplasts and 210 µl 30% PEG_3350 (polyethylene glycol MW 3350, Sigma, dissolved in 1 x SMMC and filter sterilized). PEG treatment lasted for 20 min, and phenotypic expression was carried out for 2 h in GM17S. The entire mixture was distributed onto selective plates and overlayed with 5 ml M17S soft agar (0.7% agar). After incubation for 24 h at 30°C, the plates were evalu-

ated.

Selection for Cm^r (Cm resistant), Em^r (Em resistant) and Nis^r transformants was conducted on GM17S plates at concentrations of 5, 2.5 og 100 µg pr. ml, respectively.

Competent cells of B. subtlis were transformed according to the method of Boylan et al, J. Bacteriol. 110, 281-290 (1972). Chloramphenicol (5 µg/ml) was used for the selection. To check the effects of multimeric forms of plasmids on the transformation frequencies, plasmids were linearized with suitable restriction enzymes (HindIII with pVS2, and EcoRI with pVS34) and religated before transformation , using untreated plasmids as controls.

L. plantarum and S. aureus were transformed by electroporation. A fresh inoculum using cells grown overnight was made in the appropriate broth to give an absorbance of 0.15-0.18 at 600 nm. The cultures were grown until the absorbance reached 0.5 - 0.7 and the cells harvested by centrifugation at 4°C, washed twice and finally suspended in 1/20 of culture volume electroporation buffer (272 mM sucrose, 15% glycerol). The electroporation mixture (200 µl of cell suspension held in ice for two minutes in an electroporation cuvette with a 2 mm electrode gap, to which is then added up to 20 µl purified plasmid DNA in 10 mM Tris-HCL, 1 mM EDTA, pH 7.5) was given a single electric pulse in a Genepulser ™ apparatus (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The capacitance was 25 µF and the voltage either 2 kV (L. plantarum) or 2.5 kV (S. aureus). The cuvette was connected in parallel to a 1000 ohm (L. plantarum) or 600 ohm (s. aureus) resistor (Bio-Rad Pulse Controller). The cells were then kept in ice for 2 minutes, suspended in 10 ml prewarmed culture broth, incubated for 1-2 h and harvested by centrifugation of 9 ml at room temperature, the pellet being resuspended

in the remaining 1 ml. The suspension was spread on selection plates containing either 10 µg (for L. plantarum) or 5 µg (for S. aureus) chloramphenicol per ml. L. plantarum plates were incubated anaerobically at 30°C for 48 h, while aerobic conditions and 37°C for 24-48 h were applied to S. aureus.

Plasmid elimination. Novobiocin gradient plates were used for plasmid elimination as described previously, Appl. Environ. Microbiol. 53, 1385-1386 (1987).

Southern hybridization. For the construction of pVS34, DNA was transferred to a nitrocellulose filter (Sleicher & Schuell, Dassel, FRG) using standard techniques, Leary et al., Proc. Nat'l. Acad. Sci. USA 80, 4045-4049 (1983). The biotin-labelled probe was prepared using BRL Nick Translation Reagent kit (Bethesda Research Laboratories Inc., Gaithersburg, MD, U.S.A.) and biotinylated dUTP (Boehringer GmbH, Mannheim, FRG). The staining was performed with Vectastain ABC Alkaline Phosphatase kit (Vector Laboratories Inc., Burlingame, CA, U.S.A.) according to the instructions of the manufacturer.

To demonstrate the derivation of the nisin resistance determinant, transfer of DNA from an 0.8% agarose gel (HGT agarose, SeaKem, Rockland, ME) run in TAE buffer (Maniatis) was achieved by the method of Southern, as described by Maniatis et al., to a Gene Screen nylon membrane (NEN Research Products, Boston, MA).

The template DNA for the probe was prepared by sequentially digesting about 0.6 µg pSW221 DNA with the restriction enzymes EcoRI and Bc1I, followed by electrophoresis in 0.8% agarose in TAE buffer. After excision from the gel, the largest band was purified using Gene Clean. The Boehringer Mannheim DNA Labeling and Detection

kit (Non-radioactive) was used for these purposes according to the instructions of the manufacturer.

Segregational stability of plasmids

A single colony of LM0230 carrying the plasmid in question was inoculated from a selective plate into GM17 broth, grown up overnight and then twice grown up to stationary phase from a 250-times dilution. This corresponds to growth for approximately 20 generations. The culture was then spread to single colonies on GM17 plates, and 92 clones were scored for the presence of nisin resistance (pSW211 and pSW221) or for the presence of Cm^r for pVS2 or Em^r for pGKV10.

Experimental section

Example 1A: Construction of plasmid pVS34

Lactococcus lactis subsp. lactis biovar. diacetylactis SSD207, isolated from a dairy starter culture, comprises at least 12 plasmids ranging in size from 2 to more than 30 kbp. In hybridization experiments none of the SSD207 plasmids hybridized with pVS2, a typical broad host range vector based on a 2.1 kbp lactococcal plasmid pSH71. This strain was therefore screened for plasmid replication functions with a limited capacity for expression outside the lactic acid bacteria.

Screening of SSD207 plasmid replication regions. The 4.0 kbp ClaI fragment from plasmid pVS5 (Soile Tynkkynen, M.Sc. Thesis, Univeristy of Helsinki, Helsinki, Finland) containing the chloramphenicol resistance gene of a streptococcal plasmid pGB301 (Mol. Gen. Genet. 184, 115-120 (1981)) was mixed with and ligated to the total plasmid DNA of SSD207 digested with ClaI. The ligation

mixture was used to transform MG1614 protoplasts using selection for chloramphenicol resistance.

Thirty transformants were obtained from the ligation mixture, which contained about 1 µg of DNA. The majority of the transformants carried plasmids about 8 kbp in size, while the rest apparently contained randomly inserted extra ClaI-generated fragments. One clone was arbitarily chosen for further studies, and was named pVS34. The restriction map of this plasmid is presented in Figure 1A. In DNA-DNA hybridization experiments the 4.1 kbp ClaI - BclI fragment of pVS34 (containing the replication functions) hybridized with the approximately 28 kbp plasmid of SSD207.

Location of pVS34 replication functions. To test whether any of the known restriction sites of pVS34 interrupt the replication region of the plasmid, fusion plasmids with pBR322 were generated by linearizing pBR322 with either HindIII, ClaI or BamHI and ligating to pVS34 digested with HindIII, HpaII or XhoII, respectively. E. coli was transformed with these ligation mixtures using ampicillin selection. Transformants were tested on chloramphenicol (5 µg/ml) plates. Plasmid DNA from chloramphenicol-resistant transformants were used to retransform MG1614 protoplasts. Only with the construction in which the HindIII ligation formed the hybrid plasmid were no transformants produced. With the rest, normal transformant frequencies (about 10^4 chloramphenicol-resistant clones/µg DNA) were obtained, and the plasmids extracted from the transformants had the expected sizes and restriction patterns. This, together with the successful use of the EcoRI site for cloning (see below), has led to the conclusion that at least part of the replication region of pVS34 is located within the 3.5 kbp EcoRI-ClaI restriction fragment.

Example 1B: Host range of plasmid pVS34

To determine whether the host-range of the cloned replication function, indeed, was narrow, a number of taxonomically unrelated bacteria species were attempted transformed with pVS34. In all these species it is known that the chloramphenicol resistance marker gene on pVS34 can be expressed (von Wright, A., S. Tynkkynen and M. Suominen. 1987. Cloning of a Streptococus lactis subsp. lactis chromosomal fragment associated with the ability to grow in milk. Appl. Environ. Microbiol. 53, 1584-1588).

Transformation of E. coli AB259, B. subtilis 3G18, Lb. plantarum 755, and S. aureus RN451was attempted using purified pVS34 DNA (from lactococcal preparative isolations). As positive controls parallel experiments were carried out using equal amounts of pVS2 (which contains the same chloramphenicol resistance gene as pVS34), and the transformant frequencies were compared. The results are presented in Table II. It can be seen that, besides Lactococcus MG1614, pVS34 was only able to transform Lb. plantarum and, at a very low frequency, S. aureus. The presence of pVS34 DNA could subsequently be demonstrated in Lb. plantarum and S. aureus transformants.

Table II Transformation of different recipients by plasmids pVS2 and pVS34

Plasmid	Transformant frequency / µg of plasmid DNA				
	Lactococcus lactis MG1614	E. coli AB259	B. Subtilis 3G18	S. aureus RN451	Lb. plantarum 755
pVS2	1.0×10^4	1.0×10^5	2.6×10^3	3.2×10^2	1.4×10^2
pVS2 cut and religated[a)]	-	-	2.0×10^5	-	-
pVS34	1.0×10^4	N. D.	N. D.	3.3×10^1	5.7×10^2
pVS34 cut and religated[a)]	-	-	N. D.	-	-

a) Cutting and religation were performed in order to create plasmid multimers for B. subtilis transformations (see Materials and Methods).

- = experiment not done, N. D. = no transformants detected

The amount of DNA used varied from 5 to 100 ng per transformation depending on the transformation method and recipient (optimization was done using pVS2 as a model plasmid).

Example 2A: Isolation of the nisin resistance determinant

The strain Lactococcus lactis subsp. lactis 10.084, obtained from the collection maintained at the Danish Government Research Institute for Dairy Industry, was chosen as the donor of the nisin resistance gene because of its high level of production of nisin. As a consequence of this high level, the strain is also resistant to high levels of nisin, growing unhindered in concentrations of 1000 µg/ml, and 3000 µg/ml nisin has only a slight negative effect on the bacterial growth; see Table III.

Table III. Single-cell resistance of various lactococcal strains to nisin.

	Relative number of colonies						
	concn. of nisin (µg pr. ml)						
Strain	0	10	100	500	1000	2000	3000
L. lactis subsp. *lactis* 10.084	+++	+++	+++	+++	+++	++	++
MG1614	+++	0	0	-	-	-	-
MG1614(pNis)	+++	+++	+++	+++	+++	++	++
LM0230	+++	0	0	-	-	-	-
LM0230(pVS2)	+++	0	0	-	-	-	-
LM0230(pSW211)	+++	+++	+++	+++	+++	++	++
LM0230(pSW221)	+++	+++	+++	+++	+++	++	++
MG1614(pVS40)	+++	+++	+++	+++	+++	++	++

+++: approximately same number of colonies as on GM17 plates without nisin

++: approximately one log fewer colonies as on GM17 plates without nisin

0: no colonies

-: not tested

The strain 10.084 carries 5 or 6 plasmids, and starting with the total plasmid DNA of the strain, 2 rounds of

transformation in MG1614 were carried out, followed each time by selection for nisin resistance. One of the resultant clones contained a single, 46 kbp-plasmid, which is denoted pNis. As well as being Nis^r, strain MG1614(pNis) is also Nip^+, Lac^+ and Prt^+ but cannot ferment sucrose (Table I).

The primary cloning of the nisin resistance gene from plasmid pNis was carried out by restricting about 0.8 µg pNis DNA and 2 µg pVS2 DNA with HindIII. After the vector had been treated with calf intestine phosphatase, both portions of DNA were freed of the enzymes by using Gene Clean, combined, and ligated overnight at 14°C. One-half of the ligation mixture was used to transform MG1614 followed by selection for nisin resistance. Twenty-eight transformants were screened for Cm^r and Em^r, and 26 of these showed the correct phenotype.

Eight of these clones were purified and their plasmid DNA isolated, cleaned with Gene Clean and restricted with HindIII. In all eight clones a 7.5 kbp fragment was present together with the vector. The plasmid DNA of one clone carrying only this fragment plus the vector was retransformed into LM0230. Preparative quantities of plasmid DNA, isolated via CsCl-ethidium bromide density gradients, were obtained from this clone, which is denoted LM0230 (pSW211). The phenotype of this organism (Table I and III) indicated that the cloned 7.5 kbp fragment only coded for nisin resistance and not for any of the other phenotypes of the plasmid pNis.

The restriction map of the primary, cloned fragment is shown in Figure 2A. In order to reduce the size of the nisin resistance fragment, the EcoRI fragment was cut out of an agarose gel and subcloned onto the vector pGKV10. This construction is termed pSW221 (Fig. 2B) and demon-

strates a phenotype identical to that of the entire HindIII fragment of pSW211.

As shown in Figure 3, a hybridization probe prepared from the isolated EcoRI fragment of pSW221 confirmed the presence of this fragment in one plasmid of the original wild type donor organism, in the isolated wild type plasmid pNis, and in the originally cloned HindIII fragment. This fragment showed no homology with the vector pVS2.

Example 2B: Characterization of the nisin resistance determinant

Frequency of spontaneous nisin resistant mutants in laboratory strains. Before determining the suitability of a new resistance determinant for use as a genetically selectable marker in bacteria, one must demonstrate that the bacteria naturally do not possess appreciable resistance to the compound in question and that the bacteria spontaneously will not develop any appreciable resistance. For the nisin resistance determinant in this invention, the lack of background resistance or of spontaneously arising resistance to nisin in two widely used laboratory strains was demonstrated as follows.

The frequency of spontaneous nisin resistant mutants was determined for the plasmid-free lactococcal strains MG1614 and LM0230. Cells of each strain grown in GM17 broth were harvested by centrifugation, resuspended in one-tenth the original volume sterile distilled water and plated in the appropriate dilutions on GM17 agar containing 0, 100 and 1000 µg nisin pr. ml. The plates were incubated at 30°C and the colonies counted after 24 and 48 h. The frequencies at which Nis^r colonies arise even on 100 µg pr. ml nisin are at levels normally expected for spontaneous mutations and are within the frequencies acceptable for

genetic experiments with the gene in question (Table IV).

TABLE IV. Frequency of spontaneous nisin resistant mutants arising in plasmid-free strains of L. lactis subsp. lactis after 24 and 48 h.

Strain	Period of Incubation (h)	[a] Frequency of Nis^r colonies on GM17 + nisin plates: concn. of nisin (µg pr. ml) 100	1000
MG1614	24	1.8×10^{-7}	$< 7.9 \times 10^{-10}$
	48	3.4×10^{-7}	$< 7.9 \times 10^{-10}$
LM0230	24	5.8×10^{-8}	$< 8.5 \times 10^{-10}$
	48	1.8×10^{-7}	$< 8.5 \times 10^{-10}$

[a] Values are the average of frequencies determined in 2 independent experiments. No colonies were ever observed on plates containing 1000 µg nisin pr. ml.

Occurrence of nisin resistant organisms in two starter cultures

To access the utility of the invention's Nis^r determinant in gene manipulation of organisms in dairy practice, assessment was made of the occurrence of naturally nisin resistant organisms in two actual dairy starters at present in wide use in Denmark. The two mixed strain starter cultures BOL11 and D1 were plated onto GM17 plates containing 100 and 1000 µg ml^{-1} nisin. The proportions of total cfu in the cheese starter BOL11 which were Nis^r on

100 and 1000 µg nisin pr, ml were 5.3×10^{-4} and 5.3×10^{-5}, respectively. In the butter starter D1, 3.9×10^{-1} and 1.3×10^{-1} of total cfu were resistant to nisin at 100 and 1000 µg pr. ml, respectively.

Physiological confirmation of cloning of nisin resistance determinant

The data presented in Table III illustrate the level of sensitivity to nisin of strains employed in this patent application, as well as the level of resistance endowed by the genetic marker of the present invention. These single-cell resistances to nisin confirm that the cloned HindIII fragment of pSW211, as well as its EcoRI subclone (pSW221) code for the same high level of resistance as the genome of the original wild type donor strain.

Experiments were conducted to investigate the effect of challenging with nisin exponentially growing sensitive cells and exponentially growing cells carrying the nisin resistance determinant. As illustrated in Figure 4, 10 µg pr. ml nisin in GM17 broth caused a 4 log drop in the number of viable cells in the population carrying the vector pVS2 alone [LM0230(pVS2)]. A second addition of nisin 1 ½ h after the first effected an additional drop in viable cells of at least two logs. Six h after the start of the experiment fewer than 100 viable cells pr. ml culture were detected.

In the culture carrying the HindIII fragment [LM0230(pSW211)], the initial addition of 10 µg pr. ml nisin killed about 80% of the population, and the second addition of the bacteriocin also produced a small drop in the population size.

In order to examine the genetic complement of the population inoculated with LM0230(pSW211) and still surviving 15 min. after the first addition of nisin, the phenotypes were screened of 92 colonies from the GM17 plates representing the bottom of the first drop in the curve, at 1 ¼ h (indicated by * on curve in Fig. 4). Three of the 92 clones were Nis^S Cm^S Em^S, and small-scale plasmid preparations showed that all three of these clones had lost all plasmid DNA. This result indicates that the recombinant plasmid is segregationally somewhat unstable but not more unstable than could be accounted for by the increased size of the constructed plasmids, relative to the vectors alone.

The use of the nisin resistant determinant as a selectable marker

In the following transformation experiments, the ability to select for transformants using the nisin resistance determinant of the present invention is compared with the ability to select for transformants using the traditional antibiotic resistance marker on the very same DNA molecule. The relative efficiency of selection, or selectability, of the nisin resistance determinant in transformation experiments was defined as the transformant frequency upon selection for nisin resistance relative to the transformant frequency upon selection for Cm^r or Em^r. For the recombinant plasmid pSW211 nisin selection was calculated relative to Cm^r, and for pSW221 the selection was calculated relative to Em^r. The Cm^r gene of the vector pVS2 derives from the plasmid pGB301 (Appl. Environ. Microbiol. 53, 1584-1588 (1987)), and the Em^r gene of pGKV10 is taken from pE194 (Appl. Environ. Microbiol. 50, 540-542 (1985)).

Under the conditions chosen the strain LM0230 and MG1614 are about equally competent when employed as recipients of the plasmid pVS2 (Table V). Moreover, both strains are transformed at equal but significantly lower frequencies by the 2 nisin resistant recombinant plasmids investigated in this study when selection is carried out for their Cm^r and Em^r resistances. The reductions in transformant frequencies relative to pVS2 appear to correspond with the increased sizes of the plasmids.

When selection is conducted for the Nis^r determinant itself, transformants of LM0230 are equally as efficiently selected as when the other markers on the recombinant plasmids are selected. However, when nisin resistance is selected in MG1614, transformants are only one-tenth as efficiently selected relative to the other resistance on the plasmids.

Table V. Relative efficiency of selection for the nisin resistance determinant in transformation experiments in lactococci.

Recipient	Plasmid[a]	Cm^r or Em^r transformant frequency[b] (transformants pr. µg DNA)	Relative efficiency of selection for Nis^r [c]
MG1614	pVS2	1.8×10^5	n.a.[d]
	pSW211	3.9×10^3	0.09
	pSW221	4.0×10^4	0.12
LM0230	pVS2	2.6×10^5	n.a.
	pSW211	5.9×10^3	0.86
	pSW221	3.7×10^4	1.0

[a] All plasmid DNA was isolated from LM0230 and purified through a CsCl density gradient.

[b] Cm^r and Em^r transformant frequencies are the average of at least 2 experiments and calculated on the basis of transformants arising on Cm plates (pVS2 and pSW211) or Em plates (pSW221).

[c] The relative efficiency of selection for the Nis^r determinant is defined as the transformant frequency upon selection for Nis^r divided by the transformant frequency upon selection either for Cm^r or Em^r.

[d] n.a.: not applicable.

The nisin resistance determinant is very useful as a selectable marker due to at least two properties. In the first place, the Nis^r determinant codes for a high level of resistance in the lactococci. This results in resistance-bearing strains tolerating more than 100 times as much nisin as sensitive ones. Klaenhammer and Sanozky, J. Gen. Microbiol. 131, 1531-1541 (1985), were unable to perform an actual selection for the Nis^r determinant they worked on because of a high frequency of Nis^r mutants. Although these workers also employed the strain LM0230 as a recipient, albeit, in conjugation experiments, the selection medium contained only 100 ng nisin pr. ml. This complication does not arise when using the nisin resistance determinant of the present invention due to the fact that the high level of resistance allows for direct selection of transformants at concentrations where resistant colonies arise only at frequencies typical of true spontaneous mutants.

A second quality that contributes to the suitability of the Nis^r determinant as a selectable marker is the apparently constitutive expression of the resistance. In the strain LM0230 the expression of the gene(s) does not seem to demand any more induction than the two antibiotic resistance genes used in our study as a basis of comparison of selectability. On the other hand, the strain MG1614 is only one-tenth as transformable by the Nis^r determinant as by the antibiotic resistance genes.

Effect on segregational stability of nisin resistance-encoding fragment

To investigate the extent of instability of the two nisin resistant recombinant plasmids, as well as of the vectors used in their constructions, the strain LM0230 carrying each plasmid was grown for about 20 generations in the

absence of selective pressure for the plasmids. While no clones cured of pVS2 or pGKV10 could be detected, 2.2% of the colonies phenotypically scored for pSW211 had lost the plasmid, while 4.4% of those scored for pSW221 had lost its phenotype. Indeed, plasmid preparations of the 2 clones cured of pSW211 showed no plasmid DNA, and 3 of the 4 clones cured of the pSW221-phenotype also lacked plasmid DNA. Apparently, the cloned HindIII-fragment and its EcoRI subclone have only very small negative effects on the segregational stability of the vectors on which they are cloned. This result is essential when the resistance determinant is to be used on food-grade vectors in the dairy industry. Here very large concentrations of cells are handled in the various productions and are grown in the absence of any sort of selection pressure other than that provided by milk as a growth medium.

Example 3: Construction of plasmid pVS39

The 3.7 kbp EcoRI fragment coding for nisin resistance from plasmid pSW211 was ligated to the single EcoRI site of pVS34. MG1614 protoplasts were transformed with this ligation mixture selecting for chloramphenicol resistance. Transformants were tested for nisin resistance and analysed for plasmid content. The restriction map of one of the nisin-chloramphenicol doubly resistance plasmids, denoted pVS39, is shown in Figure 1B.

Example 4: Construction of plasmid pVS40

In order to construct a nisin resistance plasmid containing only lactococcal DNA, the streptococcal chloramphenicol resistance gene was excised from pVS39. This was effected by digesting pVS39 with ClaI, isolating the 7.8 ClaI fragment containing both the nisin resistance and replication regions of pVS39 from an agarose gel and self-

ligating the fragment. This DNA was used together with similarly topologically relaxed plasmid DNA (coding for chloramphenicol resistance) to transform MG1614 protoplasts. The plasmid used in the cotransformation, pVS1, had been first linearized with ClaI, and self-ligated again. The ratio of the 7.8 kbp ClaI fragment of pVS39 to pVS1 DNA in the cotransformation mixture was about 3 to 1. Chloramphenicol resistant transformants were selected, and these were further tested for their resistance to nisin. Three doubly resistant clones were found among the twenty tested, and they all contained two plasmids corresponding in size to intact pVS1 and covalently closed supercoiled 7.8 kbp DNA. One of the clones was chosen for novobiocin curing of pVS1. Nisin (500 µg/ml) was used in both agar layers of novobiocin gradient plates. Of 100 purified single colonies picked from the novobiocin plates, one proved to be nisin resistant and chloramphenicol sensitive. It was found to contain a single plasmid representing the self-ligated 7.8 kbp ClaI fragment of pVS40 (Figure 1C).

Upon testing the segregational stability of pVS40 in the strain LM0230, it was found that 16% of the clones tested had become nisin-sensitive. This result, together with those described above in Effect on segregational stability of nisin resistance-encoding fragment for plasmids pSW211 and pSW221, would indicate that the replication function of pVS40 is not completely reliable, and that this plasmid might disappear from the bacteria population unless selective pressure for its maintenance is present. To this end, the Lac-positive construction described in Example 5 has been shown to be completely segregationally stabile when the cells are grown in a medium with lactose as the carbon source.

Example 5: Cloning of lactose fermentation gene into pVS40

The unique XhoII site of pVS40 has been successfully used in cloning the lactose fermentation genes on the BclI B-fragment of a derivative of the lactococcal plasmid pLP712 (Gasson, Hill and Anderson; Molecular genetics of metabolic traits in lactic streptococci in Ferretti and Curtiss (ed.), Streptococcal genetics, American Society for Microbiology, Washington D.C. (1987)). Isolation of Lac^+ transformants was performed by primary selection for nisin resistance, and screening on lactose-fermentation indicator plates. The Lac^+ Nis^r phenotype has been stable in further transformations with DNA isolated from the original clones. Segregationally, the recombinant plasmid between pVS40 and the Lac^+ fragment is, indeed, more stable than pVS40 itself, showing only 9% Nis^s clones as opposed to 16% for pVS40 (example 4).

P a t e n t C l a i m s :

1. A cloning vector for use in lactic acid bacteria, c h a r a c t e r i z e d by comprising a replication region with a narrow host range and a bacteriocin resistance determinant as a selectable marker.

2. The cloning vector of claim 1, c h a r a c t e r - i z e d in that the bacteriocin is nisin.

3. The cloning vector of claim 1, c h a r a c t e r - i z e d by comprising the replication region of a 28 kbp plasmid from Lactococcus lactis subsp. lactis biovar. diacetylactis SSD207.

4. The cloning vector of claim 1, c h a r a c t e r - i z e d by comprising the nisin resistant determinant from plasmid pSW211 obtained from Lactococcus lactis subsp. lactis 10.084.

5. The cloning vector of claim 1, c h a r a c t e r - i z e d by comprising the unique restriction site XhoII and further the restriction site SacI, which enables the use of insertional inactivation.

6. The lactococcal plasmid pNis or fragments thereof comprising the nisin resistance gene.

7. Lactic acid bacteria, c h a r a c t e r i z e d by being transformed with a cloning vector according to any one of the claims 1-5.

8. Plasmid pSW211.

9. Plasmid pVS34.

10. Plasmid pVS39.

11. Plasmid pVS40.

12. A nisin resistance determinant, c h a r a c t e r - i z e d by comprising a 7.5 kbp DNA fragment from Lactococcus lactis subsp. lactis 10.084.

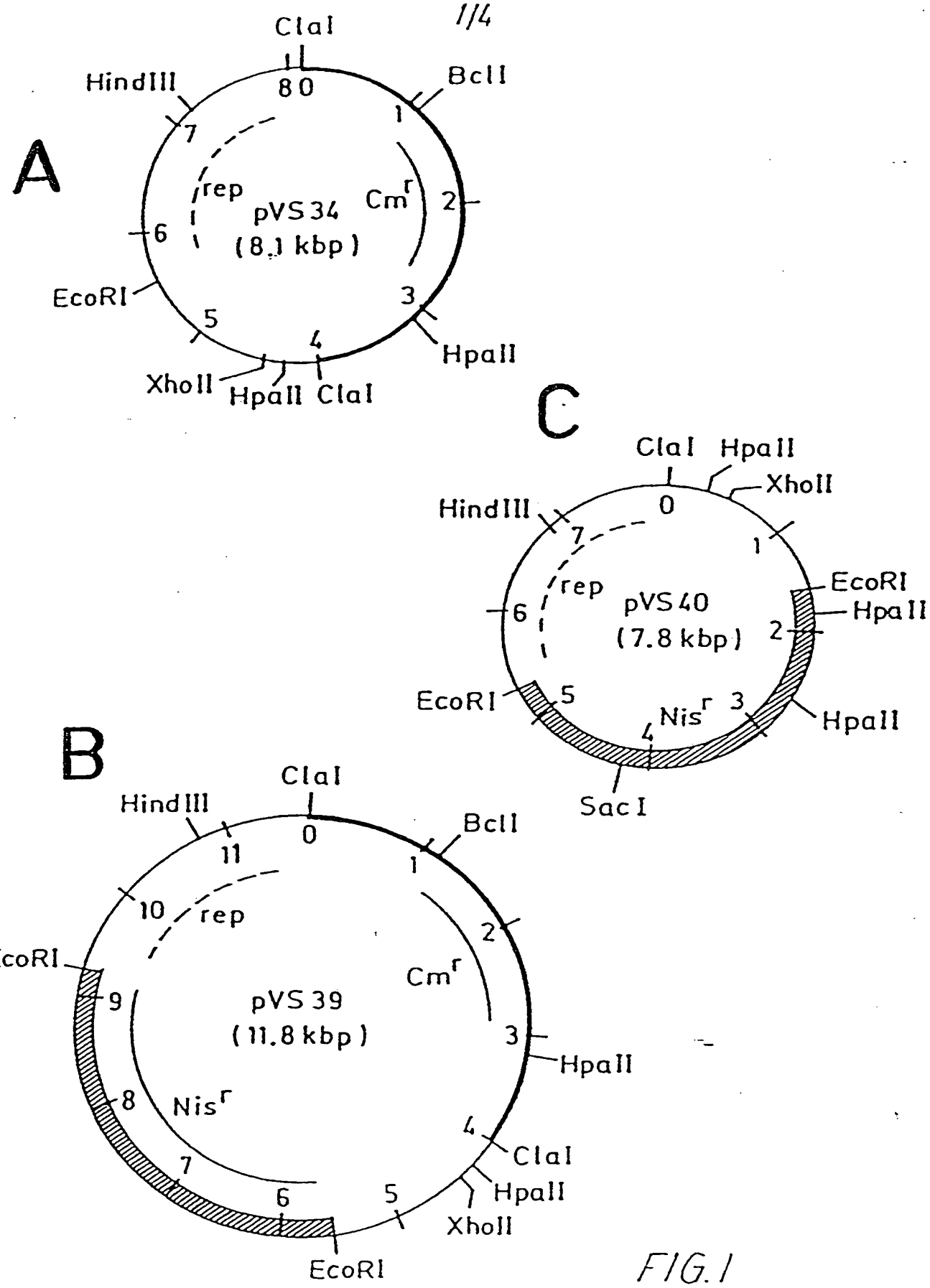

A
ClaI
BclI
HindIII
EcoRI
XhoII
HpaII
ClaI
rep
pVS34
(8.1 kbp)
Cm^r
B
ClaI
HindIII
BclI
EcoRI
HpaII
ClaI
HpaII
XhoII
EcoRI
rep
pVS39
(11.8 kbp)
Cm^r
Nis^r
C
ClaI
HpaII
XhoII
HindIII
EcoRI
HpaII
EcoRI
HpaII
SacI
rep
pVS40
(7.8 kbp)
Nis^r

FIG. 1

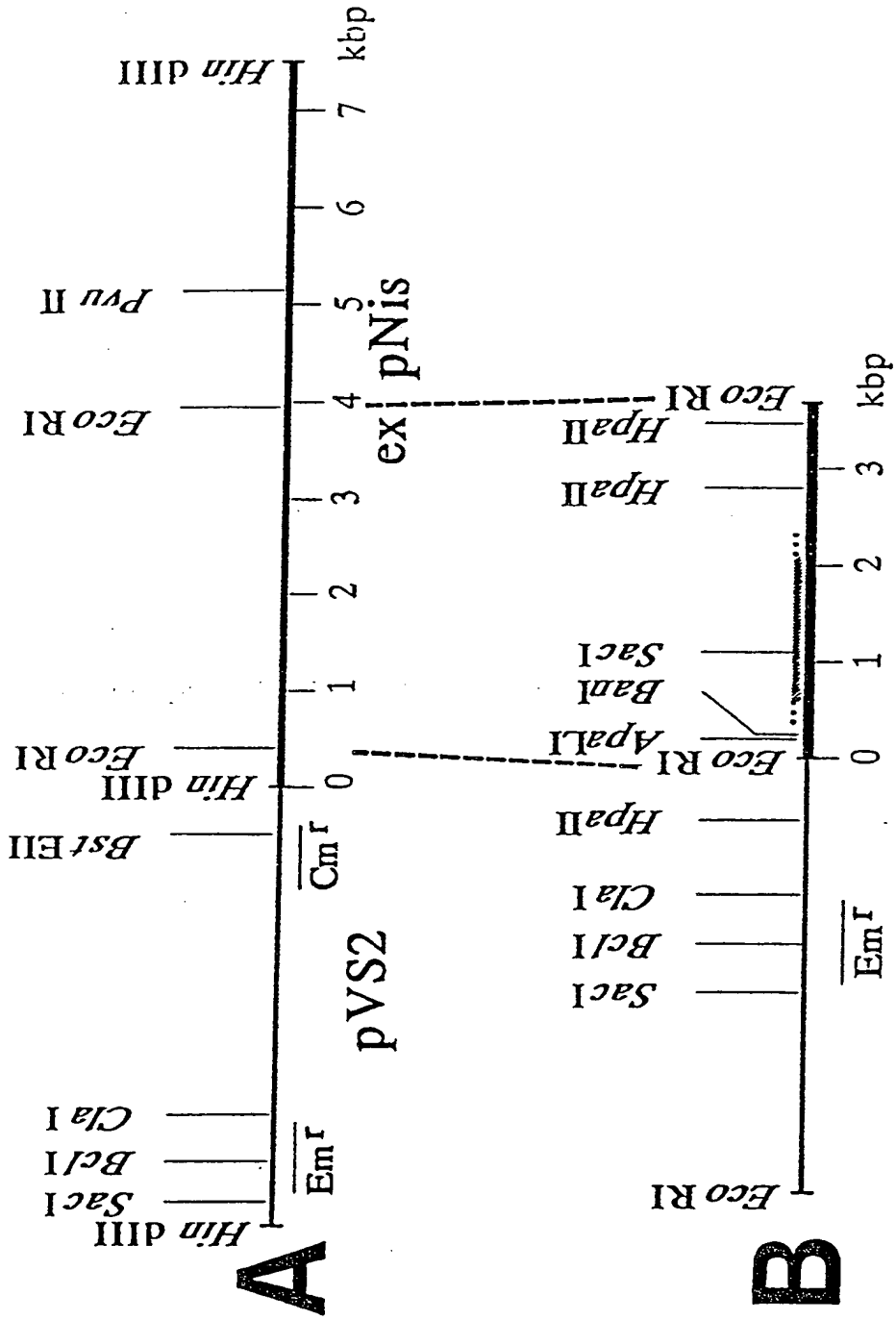

A
B
pVS2
pGKV10
pNis
ex
Hin dIII
Sac I
Bcl I
Cla I
Em r
Bst EII
Cm r
Eco RI
Pvu II
Hpa II
Apa LI
Bam I
kbp
0
1
2
3
4
5
6
7

FIG. 2

3/4

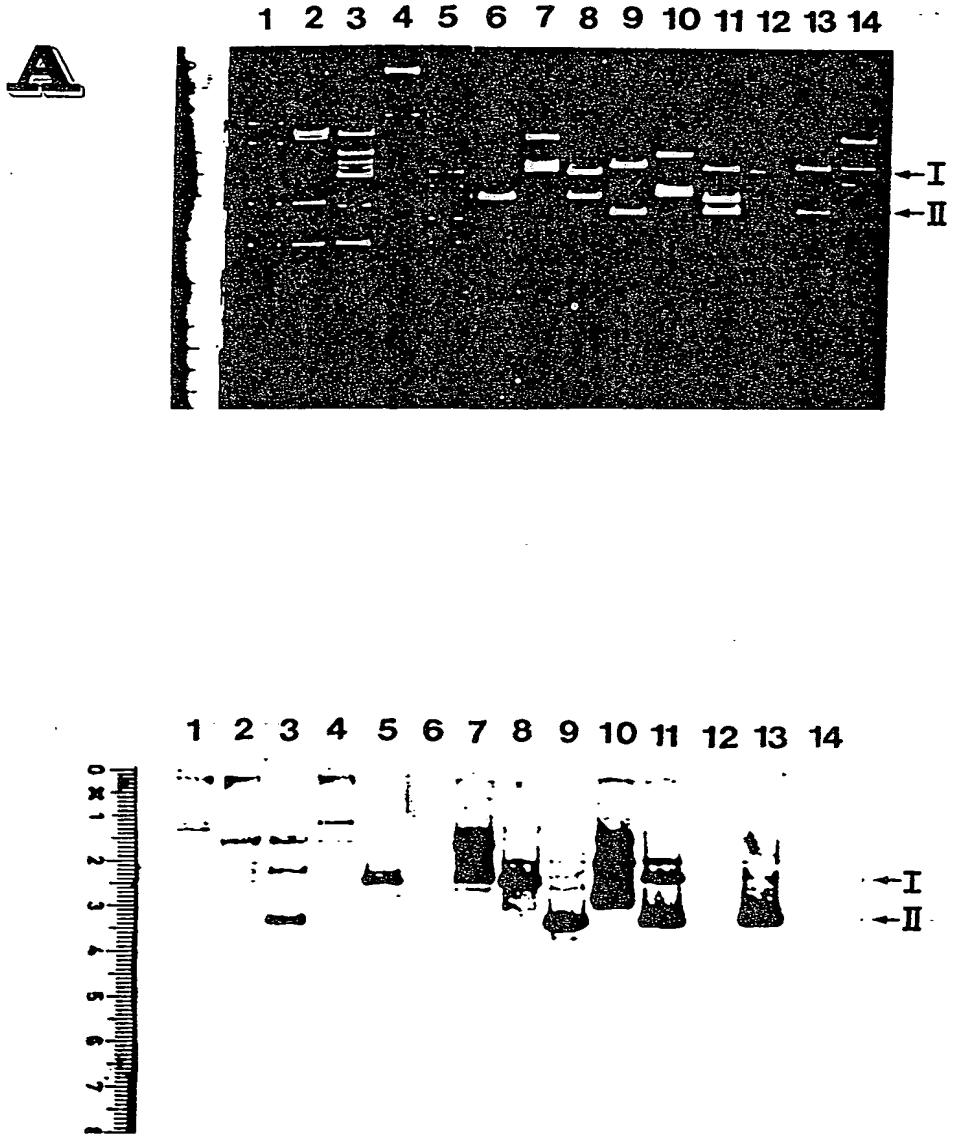


Figure 3.

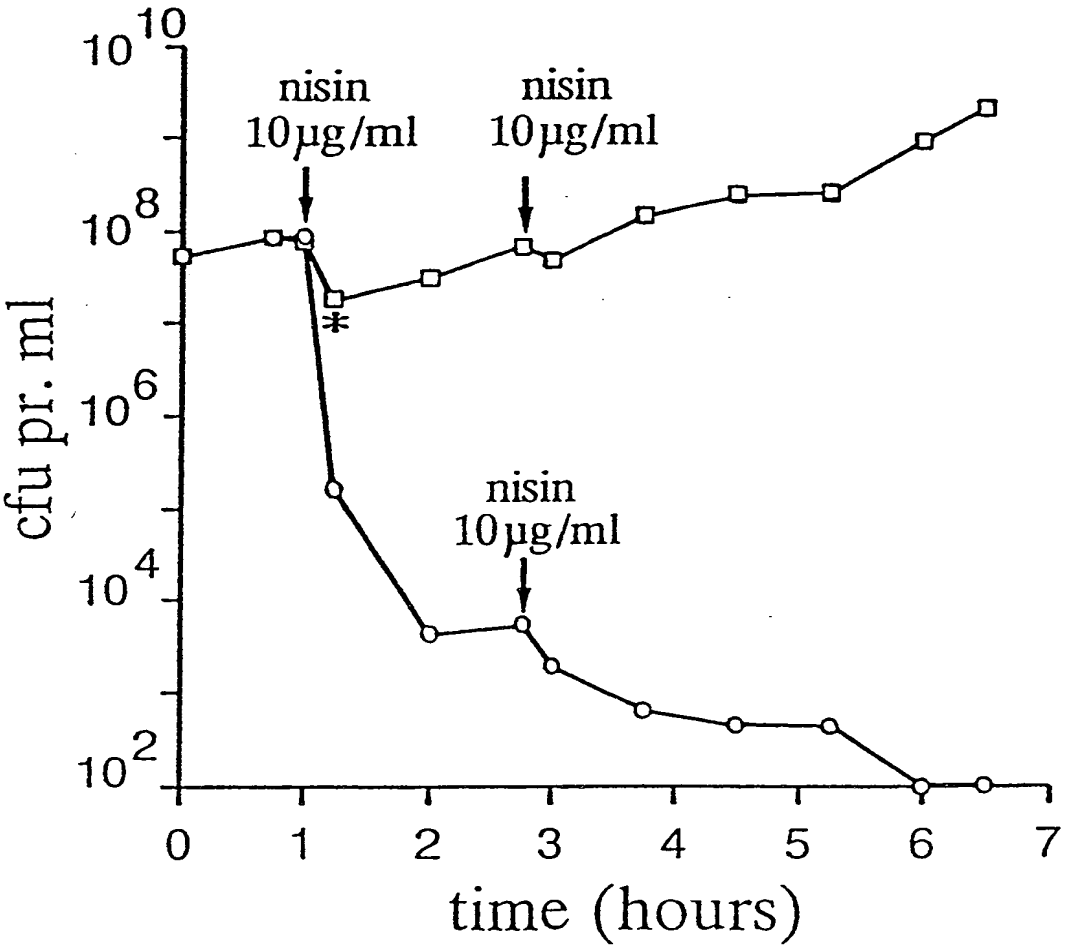

10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
cfu pr. ml
nisin
10µg/ml
nisin
10µg/ml
*
nisin
10µg/ml
0
1
2
3
4
5
6
7
time (hours)

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 89/00298

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 15/74

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	Journal of Dairy Science, Vol. 72, No. 1, July 1989 B.R. Froseth et al.: "Use of the nisin resistance determinant as a selectable marker for a food-grade cloning vector ", see page 115 --	1-12
X	EP, A1, 0228726 (STICHTING NEDERLANDS INSTITUUT VOOR ZUIVELONDERZOEK) 15 July 1987, see claims 1, 2 --	1
X	EP, A2, 0316677 (MILES INC.) 24 May 1989, see page 3, the part "Summary of the invention" --	1

----

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12th July 1990	1990 -07- 20
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Yvonne Siösteen

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 89/00298

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 90-07-04
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0228726	87-07-15	NL-A- 8503316	87-06-16
EP-A2- 0316677	89-05-24	AU-D- 2503588	89-05-18